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A Cell Surface Protein That Binds Avian Hepatitis B Virus Particles

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We have identified a 180-kDa cellular glycoprotein (gp180) that binds with high affinity to duck hepatitis B virus (DHBV) particles. The protein was detected by coprecipitating labeled duck hepatocyte proteins with virions or recombinant DHBV envelope proteins, using nonneutralizing monoclonal antibodies to the virion envelope. Binding of gp180 requires only the pre-S region of the viral large envelope protein, since recombinant fusion proteins bearing only this region efficiently coprecipitate gp180. The DHBV-gp180 interaction is blocked by two independent neutralizing monoclonal antibodies. The protein is found on both internal and surface membranes of the cell, and the species distribution of gp180 binding activity mirrors the known host range of DHBV infection. Functional gp180 is expressed in a wide variety of tissues in susceptible ducks.

Human hepatitis B virus (HBV) is a small, enveloped DNA virus that causes acute and chronic hepatitis and is strongly associated with the development of hepatocellular carcinoma (5). It is a member of the hepadnavirus group, other members of which are found in woodchucks, squirrels, and ducks; all of these agents share a common genomic organization and replicate via reverse transcription of an RNA intermediate (5, 16). These viruses are highly species specific, growing only in species closely related to their natural hosts. Within these hosts, hepadnaviruses replicate principally in hepatocytes, though low levels of extrahepatic viral DNA are detectable (6, 11, 12, 16). Little is known about the biological bases of hepadnaviral host range and tissue tropism. A major question is whether these properties are governed by virus-receptor interactions at the cell surface or by intracellular restrictions on viral gene expression (or both).

Resolution of this question will require identification of the cell surface receptor(s) for hepadnaviruses. Efforts to do this for human HBV have been hampered by the unavailability of cultured cells known to have the receptor. However, such cells are available for the duck hepatitis B virus (DHBV), in the form of primary duck hepatocytes (PDH) explanted directly from duck liver (20, 24). As a first step toward the identification of candidate hepadnaviral receptors, we have screened PDH extracts for host proteins that bind to DHBV envelope proteins. Among such molecules we might expect to find not only candidate receptors but also host factors that may participate in viral assembly, disassembly, or export. Here we report the identification of a 180-kDa duck cell membrane glycoprotein that binds DHBV envelope proteins with high affinity and specificity. Binding is inhibited by neutralizing monoclonal antibodies (MAbs) (1, 2, 25), and the species distribution of the protein mirrors the known host range of the virus.

MATERIALS AND METHODS

Plasmids. pSV45 contains the entire HBV envelope open reading frame (ORF) cloned downstream of the simian virus 40 (SV40) early promoter; it expresses all three HBV envelope proteins in roughly equal proportions and has been extensively described previously (14). pD/H was made by substituting the HBV pre-S *Bgl*II-*Eco*RI fragment of pSV45 with the pre-S *Dra*I-*Rsa*I fragment of DHBV DNA. The resulting plasmid fuses the entire DHBV pre-S region, plus the first three codons of DHBV S, to codon 4 of HBV pre-S2; the DHBV S AUG initiator codon was mutated to ACG to prevent expression from this AUG, and the plasmid was verified by DNA sequencing.

Cells, labeling, and gp180 assay. PDH were prepared from 5- to 7-day-old Pekin ducks as described previously (20) and were maintained in serum-free Dulbecco modified Eagle medium (DME) with 1.5% dimethyl sulfoxide. All other cell lines (duck and chicken embryo fibroblasts [DEF and CEF], LMH, and Cos) were maintained in DME with 10% fetal calf serum.

For radiolabeling, 100-mm-diameter plates of cells were labeled with [^{35}S]methionine (125 μCi/ml) for 4 h; after lysis in 2 ml of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride), nuclei were removed by microcentrifugation and cytoplasmic lysates (200 μl) were incubated with either (i) 5 μl of serum from uninfected or DHBV-infected ducks, (ii) 100 μl of labeled cytoplasmic extract from untransfected or transfected Cos cells, or (iii) 2 μg of the pre-S–glutathione *S*-transferase (GST) fusion protein BE1 (9) immobilized on glutathione-Sepharose. For immunoprecipitations, 2 μl of either anti-DHBV MAb 1F6 (1) or polyclonal anti-HBV surface antigen (anti-HBs; Calbiochem) was added, and immune complexes were precipitated with protein A-Sepharose and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography as described previously (14). For purification of BE1-bound complexes, 400 μl of cell extracts was added to 20 μl (2 μg) of BE1 bound to glutathione-Sepharose; these mixtures were incubated for 3 h at 4°C with rocking, washed five times with lysis buffer minus aprotinin and leupeptin, and then analyzed by SDS-PAGE and autoradiography as described above.

Cell surface biotinylation. Cos 7 cells were grown to 80% confluence on 6-cm-diameter dishes and transfected with 3.5 μg each of pSV-gp180 and pUClac9, a *lacZ* expression vector, both suspended in 1.5 ml of serum-free DME containing 300 μg of DEAE-dextran per ml. After 12 h at 37°C, cells were

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washed and incubated in DME–10% fetal calf serum (4 ml) for 48 h at 37°C. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and incubated in 1.0 ml of PBS containing 2 mg of sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce Chemical, Rockford, Ill.) per ml for 5 or 15 min. Cells were then washed three times in PBS with 100 mM glycine and incubated at 4°C for 40 min in 1 ml of the same buffer. Then the cells were washed three more times with cold PBS and lysed with 1 ml of lysis buffer; nuclei were removed by spinning for 5 min at 15,000 rpm in an Eppendorf microcentrifuge, and 400 μl of supernatant was used for precipitations as described above.

For biotinylation of total Cos cell proteins, surface-biotinylated cells were lysed in 1 ml of lysis buffer, nuclei were removed by microcentrifugation, and 200-μl samples of lysates were incubated with 5 μl of NHS-LC-biotin (2 mg/ml) for 1 h at 4°C. Then 200 μl of lysis buffer–100 mM glycine was added, and the mixture was incubated for 12 h at 4°C to terminate further biotinylation. The gp180 and LacZ proteins were precipitated as described above from 200 μl of this material.

Precipitated samples were electrophoresed on 9% polyacrylamide–SDS gels and then electroblotted onto nitrocellulose membranes.

Biotinylation of tissue extracts. For preparation of tissue extracts, 0.1 g of each tissue from a 7-day-old duckling was homogenized in 1.5 ml of lysis buffer in a Polytron for 30 s and then centrifuged for 2 min in a microcentrifuge to remove insoluble debris. For biotinylation, 200 μl of the lysate was incubated with 500 μg of NHS-LC-biotin for 1 h at 4°C. The reaction was terminated by incubation for 3 h at 4°C with 200 μl of lysis buffer containing 200 mM glycine. Biotinylated proteins were coprecipitated with Cos cell lysates expressing pD/H or pSV45 protein as described above. Samples were then electrophoresed through SDS–10% polyacrylamide gels, electroblotted onto nitrocellulose membranes, and detected by incubation with avidin conjugated to alkaline phosphatase and nitrobluetetrazolium–bromo-4-chloro-3-indolylphosphate toluidinium (salt) (NBT-BCIP) substrate.

RESULTS

Identification of DHBV-binding host proteins. To detect cellular proteins that bind DHBV, PDH cultures were prepared from the livers of uninfected 7-day-old Pekin ducklings (20, 24). After 1 to 2 days in culture, cells were labeled with [^{35}S]methionine for 4 h, and cell extracts were prepared. To these extracts was added 5 μl of viremic serum from DHBV-infected ducks; after incubation to allow virus binding, viral and subviral particles were immunoprecipitated with a non-neutralizing MAb (1F6) directed against the pre-S portion of the 37-kDa viral large (L) envelope protein (1, 25). After extensive washing of the immune precipitate, bound cellular proteins were detected by SDS-PAGE and autoradiography. As shown in Fig. 1, this MAb coprecipitated a 180-kDa radiolabeled species (hereafter termed gp180) that was not seen when serum from uninfected ducks was used. No band was seen when complexes were precipitated with anti-HBs (not shown). Identical results were obtained with virions of both European (23) and American (15) strains of DHBV (not shown). gp180 is not seen on Coomassie blue or silver stains of total cell lysates (not shown), indicating that it is a relatively minor cellular component (13) (also see Fig. 5).

gp180 binds the pre-S region of the DHBV L protein. Two related envelope proteins (L and S) are found on the surface of DHBV particles (19, 22). These proteins are encoded by a single ORF containing multiple in-frame AUG codons, of

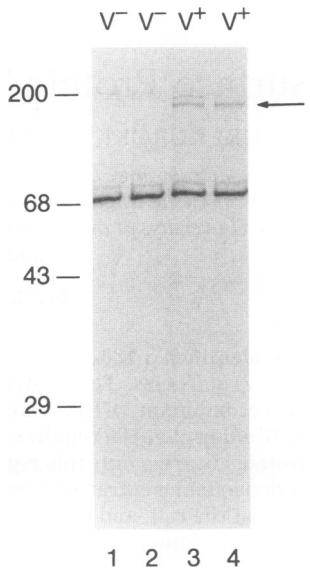

FIG. 1. A cellular protein binds DHBV particles. PDH were labeled with [^{35}S]methionine, and cytoplasmic lysates were incubated with 5 μl of serum from uninfected (V^-; lanes 1 and 2) or DHBV-infected (V^+; lanes 3 and 4) ducks for 1 h at 4°C. Then 2 μl of anti-DHBV MAb 1F6 was added, and immune complexes were precipitated with protein A-Sepharose and analyzed by SDS-PAGE and autoradiography as described previously (14). Sizes are indicated in kilodaltons.

which two are used for translational initiation. Initiation at the most distal AUG results in synthesis of the 17-kDa S protein, the predominant polypeptide of DHBV-related particles. Initiation at a more 5′ AUG generates the 37-kDa L protein, whose C-terminal 17 kDa is identical to the S protein; the unique region of L protein 5′ to the S domain is termed pre-S. To determine which of these two polypeptides is responsible for gp180 binding, we constructed the fusion gene (pD/H) shown in Fig. 2. In this chimera, the entire DHBV pre-S region is fused in frame to the HBV envelope ORF such that the DHBV pre-S domain replaces the corresponding (pre-S1) domain of HBV (Fig. 2). Expression of the DHBV-HBV chimera is driven by the SV40 early promoter. Cos 7 cells transfected with this plasmid were labeled with [^{35}S]methionine, and the viral proteins were precipitated with anti-HBs. As expected, these lysates revealed the presence of the HBV S proteins and an additional larger protein corresponding to the DHBV-HBV chimera (Fig. 2B). The latter was absent in cells transfected with control plasmid pSV45, in which the wild-type HBV envelope region was cloned behind the SV40 early promoter (14); this plasmid encodes only the three wild-type proteins of the HBV envelope (each appears as a doublet in Fig. 2, owing to glycosylation of 30 to 50% of the chains). (The polypeptides comigrating with the HBV pre-S2 proteins in lanes 3, 7, and 8 are not encoded by pD/H: they are background bands present in untransfected Cos cells [cf. lane 5].) No additional labeled bands were present in the immune precipitates from pSV45- or pD/H-transfected cells, indicating that no reactive gp180 is present in Cos cells.

Next, these labeled Cos cell extracts were admixed with [^{35}S]methionine-labeled extracts of PDH, and the resulting complexes were immunoprecipitated with anti-HBs; precipitates were then examined by SDS-PAGE (Fig. 2C). A labeled band of 180 kDa was clearly visible in PDH extracts incubated

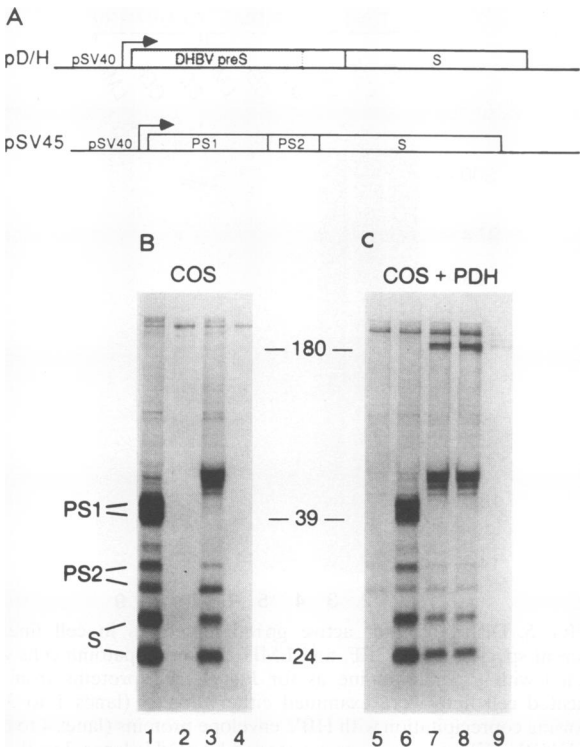

FIG. 2. (A) Schematic depiction of plasmids pD/H and pSV45, encoding the DHBV-HBV chimera and HBV envelope proteins, respectively. Vertical bars in each ORF indicate positions of in-frame AUG codons used to synthesize viral envelope proteins. For DHBV, two such AUGs are used, encoding the pre-S (L) protein and S protein; in HBV, three such AUGs are used, encoding the pre-S1 (L), pre-S2 (M), and S proteins. In both plasmids, the pre-S1 mRNA is expressed from an SV40 early promoter (pSV40), with transcription start sites indicated by arrows. Pre-S2 and S mRNAs are transcribed from promoters located within hepadnaviral sequences. (B) Expression of envelope proteins in Cos 7 cells. Cos cells were transfected with pSV45 (lanes 1 and 2) or pD/H (lanes 3 and 4), radiolabeled for 2 h with [^{35}S]methionine, then chased overnight in unlabeled methionine as described previously (14). Cell lysates (lanes 1 and 3; 100 μl per lane) or media (lanes 2 and 4) were then precipitated with anti-HBs and analyzed by SDS-PAGE. (C) Aliquots of labeled cytoplasmic extract from untransfected cells (lane 5) or from Cos cells transfected with pSV45 (lane 6) or pD/H (lanes 7 and 8) were admixed with [^{35}S]methionine-labeled PDH extracts prepared as for Fig. 1. Complexes were precipitated with anti-HBs and examined by SDS-PAGE. In lane 9, labeled PDH lysates were admixed with DHBV-positive duck serum and precipitated with MAb 1F6 as for Fig. 1. Sizes are indicated in kilodaltons.

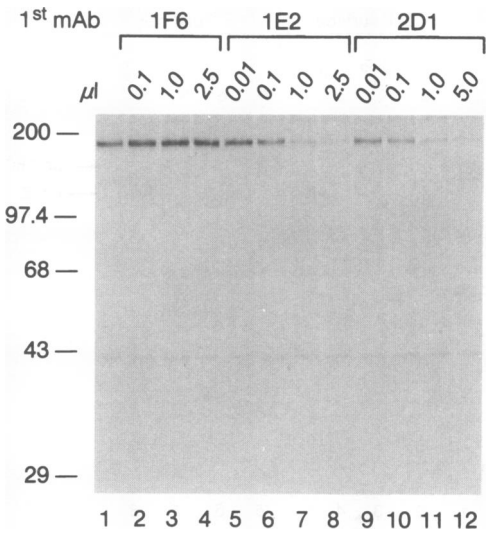

FIG. 3. gp180 binding is blocked by neutralizing but not by non-neutralizing MAbs. Labeled PDH extracts prepared as for Fig. 1 were incubated with unlabeled cell extracts from pD/H-transfected Cos cells (prepared as for Fig. 2) in the presence of the indicated volumes of MAb 1F6 (lanes 2 to 4), 1E2 (lanes 5 to 8), or 2D1 (lanes 9 to 12); 1E2 and 2D1 are neutralizing MAbs as defined by Cheung et al. (1). All of these MAbs are of the immunoglobulin G1 isotype and have comparable avidities for antigen (15a). No MAbs were added to the sample of lane 1. After 1 h at 4°C, anti-HBs (2 μl) was added, and immune complexes were precipitated with protein A-Sepharose and examined by SDS-PAGE as before. Sizes are indicated in kilodaltons.

with the DHBV-HBV fusion protein but not with the HBV proteins alone. We conclude that the binding of this cellular protein requires only the pre-S domain of the DHBV L protein. (Similar results have been obtained with DHBV and HBV pre-S regions fused to GST: only DHBV pre-S domains can bind gp180 [9].) These results accord well with indications that pre-S-containing particles can block DHBV infection (10) and that the cognate region of the HBV envelope proteins may also mediate virion binding to human hepatocyte plasma membranes (17, 18).

Neutralizing antiviral antibodies block gp180–pre-S interaction. To explore the relationship between this binding activity and viral infectivity, we next examined whether neutralizing MAbs to the virus could block DHBV binding to gp180. For this assay, we used two neutralizing MAbs (2D1 and 1E2) that map to distinct epitopes within DHBV pre-S (1, 25). Unlabeled Cos cell extracts containing the DHBV-HBV fusion protein were first incubated with increasing amounts of either a nonneutralizing (1F6) or a neutralizing (2D1 or 1E2) MAb and then mixed with labeled PDH extracts, and the resulting complexes were precipitated with anti-HBs (Fig. 3). As expected, no competition for p180 binding occurred with 1F6; however, both 2D1 and 1E2 produced a clear dose-dependent inhibition of p180 binding. (Control experiments [not shown] demonstrated that 1F6 was able to bind and precipitate the DHBV-HBV fusion protein.)

Surface expression of gp180. In studies to be reported elsewhere, we have recently succeeded in obtaining a cDNA clone for gp180 (9, 13). To facilitate studies of the subcellular distribution of gp180, we used this clone to express the protein at high levels in Cos cells. The gp180 coding region was placed under the control of the SV40 early promoter on a plasmid bearing an SV40 replication origin. This construct (pSV-gp180) was cotransfected into Cos cells together with a *lacZ* gene driven by cytomegalovirus sequences (and also bearing an SV40 replication origin). In this system, both proteins are very efficiently expressed as a result of copy number amplification of the SV40 replicon by T antigen in the Cos cell nucleus. Following transfection, intact cells (Fig. 4, lanes 1 to 4) and detergent lysates of the same cells (lanes 5 to 8) were exposed to the membrane-impermeable reagent NHS-LC-biotin (Pierce Chemical), then the biotinylation reagent was inactivated, and gp180 was precipitated with a recombinant DHBV pre-S–GST fusion protein (9). As an internal control, LacZ protein was precipitated in parallel with a specific MAb. Precipitates were fractionated by SDS-PAGE and electroblotted to solid supports; these were then probed with avidin, and

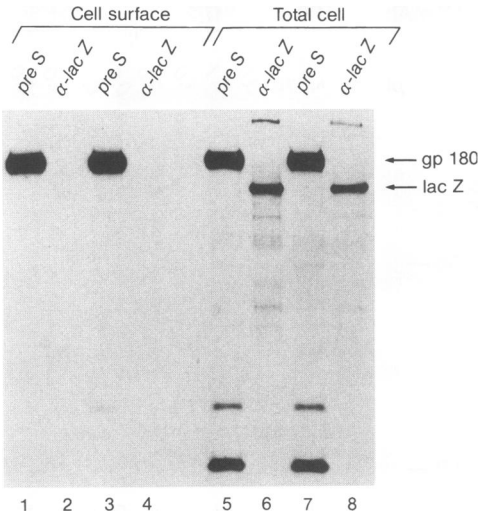

FIG. 4. Surface labeling of gp180. Cos cells were cotransfected with pSV-gp180 and pUClac9. Two days later, intact cells (lanes 1 to 4) were exposed to NHS-LC-biotin for either 5 min (lanes 1 and 2) or 15 min (lanes 3 and 4). Cell lysates prepared from these surface-labeled cells were exposed to NHS-LC-biotin for 1 h (lanes 5 to 8). Following inactivation of the labeling reagent, all samples were precipitated with either a pre-S–GST fusion protein and glutathione-Sepharose (lanes 1, 3, 5, and 7) or an anti-LacZ MAb (lanes 2, 4, 6, and 8). Precipitates were solubilized in Laemmli sample buffer, fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated avidin; bound avidin was detected the enhanced chemiluminescence method (Amersham).

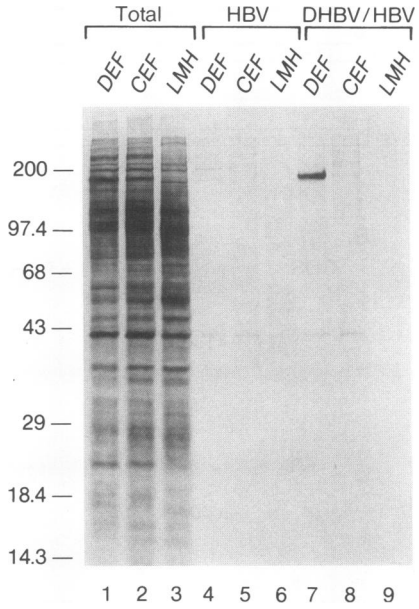

FIG. 5. Distribution of active gp180 molecules in cell lines of different species. DEF, CEF, and LMH chicken hepatoma cells were labeled with [^{35}S]methionine as for Fig. 1, and proteins from the indicated cell lines were examined either directly (lanes 1 to 3) or following coprecipitation with HBV envelope proteins (lanes 4 to 6) or the DHBV-HBV fusion protein encoded by pD/H (lanes 7 to 9). For the coprecipitations, 100 μl of unlabeled extracts from Cos cells transfected with pSV45 (lanes 4 to 6) or pD/H (lanes 7 to 9) was admixed with the indicated labeled cell lysates and precipitated with anti-HBs and protein A-Sepharose. Samples were then examined by SDS-PAGE and autoradiography. Sizes are indicated in kilodaltons.

the bound avidin was detected by enhanced chemiluminescence. As seen in Fig. 4, both proteins are readily detectable in cell lysates, but only gp180 is accessible to the surface-labeling reagant in intact cells. These results establish that gp180 is found on the cell surface. However, because of the nonlinearity of the enhanced chemiluminescence detection method, this experiment does not allow rigorous quantitation of the amount of surface protein. From other surface biotinylation experiments using PDH and colorimetric enzymatic assays to detect bound avidin, we estimate that only 5 to 10% of the total cell pool of gp180 is on the hepatocyte cell surface (9, 13).

Species and tissue distribution of gp180. We next examined a variety of cell lines of different species and tissue origin for gp180 production. [^{35}S]methionine-labeled extracts were prepared from these cell lines and admixed with unlabeled extracts of Cos cells expressing the DHBV-HBV fusion protein described above; labeled gp180 was detected by coprecipitation with anti-HBs. As shown in Fig. 5, the protein is detectable in DEF as well as PDH; however, no gp180 is found in chick CEF or in a chicken hepatoma cell line (LMH). Similarly, no binding protein was found in Cos 7 cells (simian fibroblasts; Fig. 2) or HepG2 human hepatoma cells (not shown). Since DHBV grows in ducks but not in chickens or mammals (5, 16), these results indicate that the species specificity of the binding activity mirrors the known host range of DHBV infection. It is noteworthy that while both LMH and HepG2 cells will produce DHBV particles following transfection with cloned viral DNA (3, 4, 7, 21), they do not produce viral progeny following exposure to DHBV virions. This finding suggests that these cells may lack the receptor or some other component of the entry pathway.

To assess the tissue distribution of gp180 in ducks, homogenates of individual duck tissues were biotinylated with NHS-LC-biotin and coprecipitated with the DHBV-HBV chimera, using anti-HBs. Precipitates were fractionated by SDS-PAGE and electroblotting and then probed with avidin. As shown in Fig. 6, gp180 was readily detected in every tissue examined

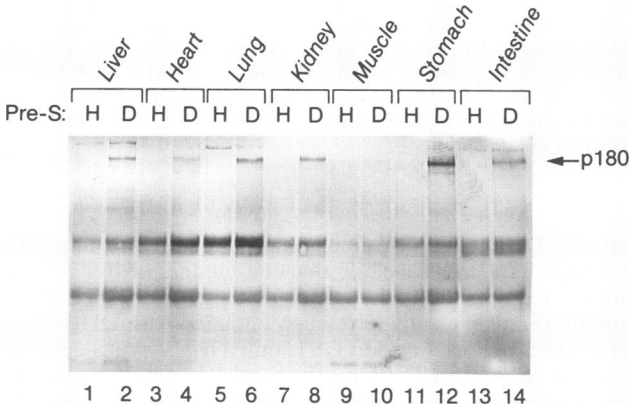

FIG. 6. Tissue distribution of gp180. Extracts of the indicated tissues were biotinylated as described in Materials and Methods. Each extract was then admixed with lysates of Cos cells expressing either pSV45 (H) or pD/H (D); complexes were then precipitated with anti-HBs antibody. Precipitated proteins were then fractionated by SDS-PAGE, transferred to nitrocellulose, incubated with avidin-alkaline phosphatase conjugates, and detected by addition of chromogenic substrate NBT-BCIP.

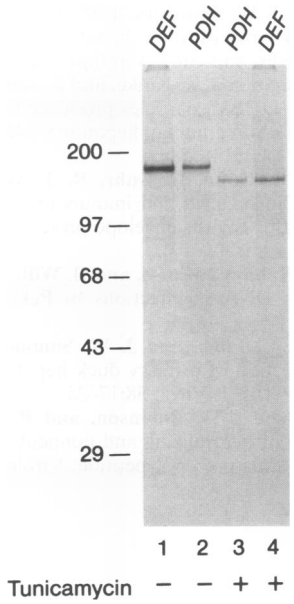

FIG. 7. gp180 is a glycoprotein. DEF (lanes 1 and 4) or PDH (lanes 2 and 3) were labeled with [^{35}S]methionine for 3 h at 37°C in the absence (−) or presence (+) of tunicamycin (1 μg/ml). Then unlabeled extracts from Cos 7 cells transfected with pD/H were added, and bound proteins were precipitated with anti-HBs and protein A-Sepharose and analyzed by SDS-PAGE. Sizes are indicated in kilodaltons.

(liver, lung, kidney, heart, stomach, and intestine) except skeletal muscle.

gp180 is a glycoprotein. That gp180 is a glycoprotein was demonstrated by labeling DEF or PDH with [^{35}S]methionine in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation (8), and then coprecipitating the binding activity with the DHBV-HBV fusion protein and anti-HBs (Fig. 7). For both cell lines, this resulted in a clear shift in the mobility of the binding activity from 180 to 150 kDa. This result also indicates that N-linked oligosaccharides are not required for the binding of gp180 to DHBV.

DISCUSSION

We have developed a biochemical assay for cellular proteins that bind hepadnaviral envelope proteins. The assay relies on coprecipitation of a prelabeled cellular factor(s) with viral envelope determinants; as such, the method is biased toward the detection of proteins whose interaction with the virus is of sufficient affinity to survive the stringent washes used in the precipitation protocol. In principle, such DHBV-binding proteins could play roles in the cell surface binding of virus, virus internalization (e.g., membrane fusion), or virion envelopment or release. We report here the first DHBV-binding protein to be identified in this fashion. This glycoprotein (gp180) is found on both internal and plasma membranes of many different avian tissues and binds specifically to the pre-S region of the DHBV L protein. This binding is blocked by neutralizing but not nonneutralizing MAbs and has a species distribution similar to the viral host range.

All of these properties are most consistent with a role for gp180 in the DHBV entry pathway. We think it unlikely that the protein plays an essential role in virion assembly or egress, since functional protein is missing from LMH cells, which are nonetheless capable of generating and secreting infectious DHBV following transfection with cloned viral DNA (3). (This does not, of course, exclude a stimulatory or other regulatory role for gp180 in such steps.) The simplest model that unites most of these observations would be that gp180 is the DHBV receptor. However, we emphasize that all of these arguments are circumstantial, and some of our other observations are not simply reconciled with this model. Chief among these is the presence of gp180 in normal yields on a variety of tissues and cell lines (e.g., DEF) that are not susceptible to DHBV infection. Several interpretations are compatible with this fact: (i) gp180 might be only one subunit of the receptor, in which case these cells would lack another essential subunit; (ii) gp180 is the receptor, but postreceptor blocks to infection exist in such cells; (iii) gp180 itself acts at a postreceptor step, and the true receptor is missing from these nonpermissive cells; or (iv) gp180 has nothing whatsoever to do with viral entry. Further experiments are in progress to examine these possibilities in more detail. Ultimately, proof of a causal role for gp180 in entry will require demonstration that antibodies to this protein block PDH infection or that expression of the clone in LMH or HepG2 cells, which are nonpermissive for virion infection, confers susceptibility to infection by DHBV particles.

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